

10/23/00
Jc946 U.S. PTO

10-24-00

A

FORM PTO-1082

82425.0055
Express Mail Label No. EL589806195US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
Edward Shanbrom
Serial No: Not Assigned
Filed: October 23, 2000
For: ENHANCERS FOR MICROBIOLOGICAL DISINFECTION

Jc915 U.S. PTO
09/694178
10/23/00

Box PATENT APPLICATION
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Transmitted herewith for filing is the patent application identified above.

- ☒ 0 sheet(s) of drawings (☐ formal ☐ informal) is(are) enclosed.
☒ 25 page(s) of specification and 1 page of abstract of the invention are enclosed.
☒ Return postcard is enclosed.
☒ copy of provisional patent application which was filed October 20, 2000 from which priority is claimed in the subject case pursuant to 35 U.S.C. § 119
☒ Assertion of Small Entity Status Under Section 1.27.
☒ Check in the amount \$355 for filing fee.
☒ Certificate of Express Mailing.

CALCULATION OF FEES								
ITEM		TOTAL NO. OF CLAIMS		NO. OF CLAIMS OVER BASE	LG/SM \$ ENTITY FEE		\$ AMOUNT	\$ FEE
A	TOTAL CLAIMS FE E	16	-20	20	LG=\$18 SM=\$9	\$0	0	
B	INDEPENDENT CLAIMS FEE*	4	-3	1	LG=\$80 SM=\$40	\$40	40	
C	SUBTOTAL - ADDITIONAL CLAIMS FEE (ADD FINAL COLUMN IN LINES A + B)							40
D	MULTIPLE-DEPENDENT CLAIMS FEE				LARGE ENTITY FEE = \$270 SMALL ENTITY FEE = \$135			\$ 0
E	BASIC FEE				LARGE ENTITY FEE = \$710 SMALL ENTITY FEE = \$355			\$ 355
F	TOTAL FILING FEE (ADD TOTALS FOR LINES C, D, AND E)							\$ 395
G	ASSIGNMENT RECORDING FEE							
	*LIST INDEPENDENT CLAIMS [LIST OF INDEPENDENT CLAIMS]1, 4, 9, 14							

- ☒ Please charge my Deposit Account No. 50-1314 the amount of \$ 40.00. A copy of this letter is enclosed.
☒ A check in the amount of \$ 355.00 to cover the filing fee is enclosed.
☐ A check in the amount of \$__ to cover Assignment Recordation fee is enclosed.
☒ The Commissioner is hereby authorized to charge any deficiency for the following fees associated with this communication or credit any overpayment to Deposit Account No. 50-1314. **A copy of this sheet is enclosed.**
☒ Any additional filing fees required under 37 C.F.R. 1.16
☒ Any patent application processing fees under 37 C.F.R. 1.17
☒ The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. 50-1314. **A copy of this letter is enclosed.**
☒ Any patent application processing fees under 37 C.F.R. 1.17
☐ The issue fee set in 37 C.F.R. 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b)
☒ Any filing fees under 37 C.F.R. 1.16 for presentation of extra claims
Please associate this case with the attorneys of record.

Date: October 23, 2000

500 South Grand Avenue, Suite 1900
Los Angeles, CA 90071
Telephone: (213) 337-6700
Facsimile: (213) 337-6701

Respectfully submitted,
HOGAN & HARTSON L.L.P.

By:

Stefan J. Kirchanski
Stefan J. Kirchanski
Registration No. 36,568
Attorney for Applicant(s)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Edward Shanbrom

Serial No: Not Assigned

Filed: October 23, 2000

For: ENHANCERS FOR MICROBIOLOGICAL
DISINFECTION

Art Unit: Not Assigned

Examiner: Not Assigned



CERTIFICATE OF MAILING VIA U.S. EXPRESS MAIL

"Express Mail" Mailing Label No. EL589806195US

Date of Deposit: October 23, 2000

Box PATENT APPLICATION

Commissioner for Patents

Washington, D.C. 20231

Dear Sir:

I hereby certify that

- ☒ two copies of a letter of transmittal
- ☒ check in amount of \$ 355 as filing fee
- ☒ patent application (25 page(s) of specification; 16 claim(s); 1 page of abstract
- ☒ Assertion of Small Entity Status Under Section 1.27
- ☐ copy of provisional patent application which was filed October 20, 2000 from which priority is claimed in the subject case pursuant to 35 U.S.C. § 119
- ☒ return postcard

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service with sufficient postage under 37 C.F.R. § 1.10 on the date indicated above and are addressed to:

Box PATENT APPLICATION

Commissioner for Patents

Washington, D.C. 20231.

Date: October 23, 2000

Hogan & Hartson, LLP
500 South Grand Avenue, Suite 1900
Los Angeles, California 90071
Telephone: 213-337-6700
Facsimile: 213-337-6701

Darin Sutton
Name of person mailing papers

[Signature]
Signature

L.L.P.

BILTMORE TOWER
500 SOUTH GRAND AVENUE, SUITE 1900
LOS ANGELES, CALIFORNIA 90071
TEL: (213) 337-6700
FAX: (213) 337-6701
WWW.HHLAW.COM

**Affiliated Office*

general info		age group		sex		ethnicity		education		employment		marital status		religion		health status		social support		stress level		coping strategies		quality of life					
name	id	age	gender	race	ethnicity	education	employment	marital status	religion	health status	social support	stress level	coping strategies	quality of life	name	id	age	gender	race	ethnicity	education	employment	marital status	religion	health status	social support	stress level	coping strategies	quality of life
John Doe	101	45	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Jane Smith	102	35	Female	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Robert Johnson	103	55	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Emily White	104	25	Female	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Michael Brown	105	65	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	David Green	106	45	Male	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Sarah Lee	107	35	Female	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Christopher Hill	108	55	Male	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
James Wilson	109	45	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Amanda Taylor	110	35	Female	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Patricia King	111	55	Female	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Benjamin Clark	112	65	Male	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
William Davis	113	45	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Elizabeth Adams	114	35	Female	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Linda Miller	115	55	Female	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Thomas Evans	116	45	Male	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Richard Scott	117	65	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Michelle Baker	118	35	Female	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Barbara Hall	119	45	Female	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Gregory Nelson	120	55	Male	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Kevin Carter	121	35	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Angela Perez	122	65	Female	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Christopher Evans	123	45	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Stephanie King	124	55	Female	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Michelle Roberts	125	35	Female	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Jonathan Lee	126	65	Male	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Anthony Walker	127	45	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Victoria Young	128	35	Female	White	Hispanic	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Deborah Allen	129	55	Female	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Timothy Wright	130	45	Male	Black	African American	College	Employed	Single	Protestant	Fair	High	Emotional Support	7.0	
Gregory King	131	35	Male	White	Hispanic	High School	Unemployed	Married	Catholic	Good	Low	High	Problem Solving	8.5	Christina Lopez	132	65	Female	White	Hispanic	College								

Assigned to

for

prepared by:

Attorney Docket No. 82425.0055

Enhancers for Microbiological Disinfection

Background of the Invention

Area of the Art

5 The present invention deals with disinfection in the medical area and in particular agents that greatly potentiate the activity of known antimicrobial and disinfection agents.

Description of the Prior Art

10 It has long been known that various chemical agents can be used to kill or otherwise limit the growth of infectious agents such as bacteria and viruses. Many of the initial disinfecting materials were active, even caustic, chemicals such as the "carbolic acid" (phenol) used by Lister in the early days of the germ theory of disease. In the last century anti-infective agents of biological origin such as penicillin, were discovered and widely exploited. Antibiotic drugs used to
15 treat infectious diseases have proliferated and were used liberally in developed countries following the Second World War. These drugs are widely used not only to treat disease but to obtain enhanced growth of farm animals living under often substandard "factory farm" conditions. As a result of this sort of excessive use and misuse by patients, many new, antibiotic resistant organisms have evolved.
20 Drug companies and researchers are actively seeking new compounds to fight these resistant disease organisms. Most of their research however is targeted at finding single chemical compounds or combinations of chemical compounds that act on these organisms.

25 Combination therapies have been around for many years as demonstrated in the book "Combination Antibiotic Therapy in the Compromised Host", edited by J. Klastersky and MJ Staquet, New York, Raven Press, 1982. Other more

recent work on combination therapies is the work done with fluoroquinolones (Meyer, RD, J. Antimicrob. Chemother., 1991, 28, 623). The general idea of combination therapy is to develop a synergistic drug combination or to somehow enhance the activity of a known antibiotic or even some other disinfectant or anti-infective agent. In many instances combination therapies are sought as ways to overcome drug resistant disease organisms. For example, penicillin, the first widely used antibiotic, has now lost much of its usefulness due to resistant organisms. One form of penicillin resistance involves the production of penicillin destroying enzymes (penicillinase) by disease organisms. Therefore, one type of combination therapy is to combine compounds that inhibit penicillinase with the penicillin. This approach can largely restore the resistant organism's sensitivity to penicillin.

Antibiotics are natural biological compounds often of bacterial or fungal origin. Many of our drugs are of plant origin. Therefore, it is hardly surprising that much research is now being conducted in the area of the antimicrobial qualities of herbs, fruit extracts and other materials of plant origin. For the most part, companies and researchers are studying the efficacy of extracts of plant materials, alone or the efficacy of specific purified molecules of the plant material extracts. A series of patents by Walker et al. (US Patent Numbers 5,474,774, 5,525,341, 5,646,178, and 5,650,432) describe the isolation and purification of specific compounds from cranberry for use as antimicrobials. In particular it was demonstrated that these agents interfere with the adhesion of bacteria to the endothelial cells of the urinary tract. The present inventor has purified antimicrobial colored compounds from cranberry and other plant materials. These compounds actually have bacteriostatic as well as bactericidal and virucidal activities as described in U.S. Patent Number 6,093,401.

The present inventor has long had an interest in disinfecting blood and blood products. Out of this interest the just mentioned interest in antimicrobial

plant products developed. From his many years of experience with blood fractionation and production of blood products the present inventor was extremely familiar with the use of various chemicals as anticoagulants in blood and blood products. Common anticoagulants are EDTA, heparin, oxalic acid, citric acid and their salts. Of these all but heparin function primarily by reducing the level of calcium ions. One of the inventor's experiments aimed at disinfecting blood plasma inadvertently removed the citric acid (actually sodium citrate) used as an anticoagulant with the resulting precipitation (coagulation) of the plasma. Attempts were made to overcome this problem through the addition of extra citric acid. This resulted in the present discovery of the unexpected properties of citric acid, which forms the substance of the present invention.

It should be appreciated that there is a pressing need for the disinfection of blood and blood products used for transfusion and other medical therapies. In the first place collected blood is virtually always contaminated with bacteria commonly present on the skin of the blood donor. The needle used for blood donation picks up these organisms and contributes them to the donated blood. These bacteria especially cause problems with platelet concentrates whose useful life is currently limited to five days because of bacterial growth whereas the biologically useful life of the concentrates is at least several additional days. With the current shortages of donated blood this rapid outdateding of platelet concentrates is especially troublesome.

Another reason for disinfection is the one that occurs to most people concerned with blood safety—namely blood borne disease. When the blood donor is infected with viral or other disease agents, the infection may be spread through blood transfusion or receipt of blood products made from infected blood. Currently a detergent-based process, originated by the present inventor, is used to inactivate many viruses present in blood plasma. This results in safer plasma and blood products made therefrom. However, detergents cannot be used on

whole blood because they damage or destroy blood cells. Thus, there is a great need for methods to disinfect whole blood and cellular fractions thereof. Further, the current detergent process does not inactivate all dangerous blood borne viruses. Therefore, there is also a pressing need for improved disinfection of plasma.

The present inventor has discovered that increasing the concentration of citrate to levels higher than those usual for anticoagulation results in significant decreases in bacteria and bacterial growth in blood and blood fractions. Further, citrate shows an unexpected synergistic effect when combined with a wide variety of antibiotics, antimicrobials and disinfecting agents including iodine and natural plant products such as those from grape of cranberry. Citrate has been used in dental rinses and is widely used as an anticoagulant and a buffering agent in foods, pharmaceuticals and cleaning materials. However, the antimicrobial synergistic effects of citrate have hitherto not been appreciated.

Summary of the Invention

Simple carboxylic acids, in particular dicarboxylic acids such as citric acid shows an unexpected ability to enhance the antimicrobial power of a wide range of disinfectant and/or antibiotic agents. As little as 1% citrate greatly enhances the ability of antibiotics to kill or inhibit a wide range of bacterial species including antibiotic resistant strains. Citrate alone is effective in preventing bacterial growth in platelet concentrates and in red blood cell suspensions. Effective concentrations of citrate cause little if any damage to blood cells. Besides enhancing the power of antibiotics citrate also enhances the antimicrobial properties of disinfectant organic dyes such as crystal violet and methylene blue. In addition citrate enhances the antimicrobial properties of polyphenols of plant origin. Iodine-based disinfectants are also enhanced without enhancing protein denaturation.

Detailed Description of the Invention

The following description is provided to enable any person skilled in the art to make and use the invention and sets forth the best modes contemplated by the inventor of carrying out his invention. Various modifications, however, will remain readily apparent to those skilled in the art, since the general principles of the present invention have been defined herein specifically to provide a method to greatly enhance the antimicrobial effects of antibiotics, plant natural products and disinfecting chemicals.

Citrate Effect on Platelets and Red Cells

Two 50 ml aliquots of platelet rich plasma were prepared by centrifugation. One of the samples was brought to 2% by weight citric acid As explained above, human platelet concentrates are ordinarily contaminated with human skin bacteria. These bacteria grow even if the platelet concentrates are kept at low temperatures. Such bacterial growth significantly shortens the useful life of these platelet preparations. In this experiment the solution were incubated on a rotator at room temperature. Such conditions are ideal for preservation of platelet function but are also excellent for bacterial growth. Samples were removed each day and counted to determine the state of the platelets. It was already determined in other experiments that these levels of citrate were very effective in killing or inhibiting bacteria. The question was whether the citrate would harm the platelets.

Treatment Day	Platelet Count (per ml) (control)	Platelet Count (per ml) (2% Citrate)
1	4.0×10^5	4.2×10^5
2	4.2×10^5	4.3×10^5
3	3.7×10^5	4.0×10^5

Treatment Day	Platelet Count (per ml) (control)	Platelet Count (per ml) (2% Citrate)
4	3.8×10^5	3.7×10^5
5	3.7×10^5	3.7×10^5
6	2.7×10^5	3.1×10^5

These results indicate that platelets do not show obvious damage after exposure to 2% citrate. The platelet counts appear to be about the same either with or without the citrate. Over time the number of platelets gradually decreases. However, the decrease is slower in the presence of citrate suggesting that this agent may actually stabilize or preserve the platelets. Other studies have shown that platelets maintain adequate function for at least seven days, but solutions are not used beyond five days because of excessive bacterial growth.

Similarly, 2% citrate does not appear to damage red blood cells (RBCs). Normally red cells are very sensitive to added agents. The damage to the red cell membrane can be observed as hemolysis (leakage of hemoglobin from the cells) or as an increase in potassium (K^+) or lactate dehydrogenase (LDH) both of which leak out of damaged red cells. In this experiment blood sample either with or without citrate (2%) were sampled over a seven-day period.

Control

Day	Hemolysis	K^+ ($\mu\text{g/ml}$)	LDH (IU/ml)
1	—	3.2	100
2	—	3.2	101
3	—	3.3	100
4	—	3.2	102
5	—	3.4	101

Day	Hemolysis	K ⁺ (μg/ml)	LDH (IU/ml)
6	—	3.4	103
7	slight	3.5	105

2% Citrate

Day	Hemolysis	K ⁺ (μg/ml)	LDH (IU/ml)
1	—	3.2	101
2	—	3.3	100
3	—	3.3	100
4	—	3.3	100
5	—	3.2	101
6	slight	3.5	106
7	slight/ moderate	3.8	110

These results indicate that increased citrate may have a very slight effect on red cells (increased because 0.5% citrate is commonly used as an anticoagulant). However, this is much less harmful than many other agents that have been used in an attempt to disinfect red cells. Therefore, citrate can be combined with other agents to disinfect red cells with increasing the damage to the red cells. An important point is that the added citrate can be readily reduced or removed with anion exchange materials.

Citrate and Antibiotics

The effects of 5% and 8% (weight by volume) of citric acid on bacteriological broth was investigated. In each case the pH of the broth was adjusted with NaOH to bring it into normal growth ranges if necessary. The amount of growth is rated from minimum (+) to maximum (4+). If no growth occurred, the treatment is marked 'NG'. Each broth sample was inoculated with

1x10³ organisms of vancomycin resistant *Enterococcus*. The inoculated broth was incubated for 2 hr at 35 °C for 2 hr and then plated on growth media, which was allowed to incubate overnight under optimal growth conditions.

Medium	Level of Growth
Broth alone	4+
Broth + 5% citrate	4+
Broth + 8% citrate	3+
Broth + 250 µg/ml vancomycin	3+
Broth + 5% citrate + 250 µg/ml vancomycin	1+
Broth + 8% citrate + 250 µg/ml vancomycin	NG

5 These results show that either 8% citrate or 250 µg/ml vancomycin alone cause some inhibition of bacterial growth. This level of the vancomycin was selected because it would be sufficient to completely inhibit non-resistant bacteria. The combination of citrate with the vancomycin completely inhibits the resistant bacteria. This shows that the synergistic effect of vancomycin with
10 citrate is sufficient to overcome bacterial resistance.

Citrate is also effective with other combinations of bacteria/antibiotics. In the following experiment a suspension of 1x10³ cell/ml of *Klebsiella pneumoniae* was prepared in trypticase soy broth. Ten ml aliquots were treated with citrate and/or antibiotics. After a two hr incubation at room temperature, 0.1 ml samples
15 of each treatment were spread on soy trypticase broth agar and incubated overnight at 35 °C. The plates were then scored for bacterial growth with the following results:

Medium	Level of Growth
Broth alone	4+
Broth + 2% (by wgt.) trisodium citrate	3+
Broth + 8% citrate	3+
Broth + 25 µg/ml ampicillin	2+
Broth + 2% trisodium citrate + 25 µg/ml ampicillin	NG

These results indicate that the antibiotic-citrate effect extends to more than a single antibiotic-bacterial species combination. Additional experiments (data not shown) have demonstrated that citrate enhances the activity of a wide range of antibiotics against a large number of bacterial species and strains both wild types and antibiotic resistant types.

Citrate Enhancement of Antimicrobial Pigments

It has long been known that a variety of organic dyes show antimicrobial properties. Much study was expended on these compounds prior to the discovery of modern antibiotics. Some of the "disinfectant" dyes such as crystal (gentian) violet still enjoy some use. I have discovered in other work that a combination of two of the disinfectant dyes—namely crystal violet plus methylene blue—are especially effective at preventing growth of a wide range of bacteria. I have also previously discovered that the polyphenol pigments which precipitate during the aging of grape wine (called lees) also exhibit antibacterial properties. The following experiment was carried out to see if the synergistic properties of citrate extend to such antimicrobial pigments. There is some indication in the literature that ascorbic acid may also show some synergistic properties with such antimicrobials. Therefore, sodium ascorbate was also tested.

An aqueous solution of equal weights of methylene blue and crystal violet (0.0057% of each) was prepared. Also an aqueous suspension of red wine lees (0.25%) was also prepared. Treatment solutions were prepared by adding either

sodium citrate or sodium ascorbate to 1.5% by weight from a stock solution.
Control aliquots contained neither citrate nor ascorbate.

5 For test purposes each sample was inoculated with 1×10^4 cells of either *Escherichia coli* or *Staphylococcus epidermidis*. The sample was then incubated at room temperature for 60 min with agitation. At the end of the incubation period the samples were plated onto TSA growth media and incubated overnight at 35°C. The plates were then scored for number of colonies with NG indicating “no growth” and 4+ indicating maximal growth.

Treatment	<i>E. coli</i>	<i>S. epidermidis</i>
Control	4+	3+
Dye alone	2+	2+
Citrate alone	1+	----
Dye-citrate	NG	NG
Dye-ascorbate	1+	1+
Lees alone	1+	2+
Lees-citrate	NG	NG
Lees-ascorbate	1+	2+

10 These results indicate that both the dye mixture and the lees show significant antibacterial properties. When either dye or lees are combined with 1.5% sodium citrate all bacterial cells are killed or prevented from further growth. Ascorbate shows some ability to potentiate the effect of the dye mixture but has no apparent effect on the lees.

Citrate Compared to EDTA

EDTA (ethylenediaminetetraacetic acid) is a calcium chelating anticoagulant that is known to have some antimicrobial activity. Sodium diacetate is another calcium chelating material with known antimicrobial and anticoagulation properties. The chelating agents were compared by incubating suspension of 1×10^3 cells/ml of *Escherichia coli* or *Staphylococcus epidermidis* in human plasma for 60 min at room temperature for 60 min prior to incubating 100 μ l of suspension (potentially 100 cells) under optimal conditions for 12 hrs. Human plasma represents a rich growth medium and heavy bacterial growth is expected. All added compounds are expressed as percent by weight.

Treatment	Growth <i>S. epidermidis</i>	Growth <i>E. coli</i>
Control (plasma alone)	4+	4+
8% Sodium EDTA	---	3+
8% Sodium Diacetate	---	NG
8% Sodium Citrate	NG	NG
2% Sodium Citrate	---	1+
8% Citric Acid	NG	NG
8% Sodium Oxalate	3+	3+
8% Sodium Heparin	3+	3+

These results that EDTA, sodium oxalate and sodium heparin even at concentration considerably higher than used for anticoagulation shows limited antibacterial properties. At the high level of 8% sodium diacetate completely prevents growth of *E. coli*. At 8% both sodium citrate and citric acid prevent bacterial growth entirely. At 2% sodium citrate causes considerable inhibition of

E. coli. It should be kept in mind that this percentage is still some four times the normal amount used for anticoagulation. Since the anticoagulating ability of both EDTA, oxalate and citrate are related to the ability of these compounds to chelate calcium ions, it seems apparent that the antibacterial effect of citrate is not due to its effect on calcium ions or at least not solely to its effect on calcium ion. Like citrate the diacetate has multiple carboxylic acid groups per molecule.

Citrate Effect on Povidone Iodine

A 50 ml aliquot of 2% (by weight) trisodium citrate in bacterial growth broth was prepared and spiked with *Escherichia coli* (1×10^8 cells/ml). The spiked broth was divided into six 8 ml aliquots. A 25 ml aliquot of spiked broth without citrate was used as a control. The control aliquot was divided into three 8 ml samples. Samples were taken at 30 min, 6 hr and 24 hr while the aliquots incubated at room temperature. For the iodine samples 400 μ l of 10% povidone-iodine (PVP-I) was added to the sample 5 min prior to the sampling. The samples were plated on trypticase soy agar (TSA) and allowed to grow overnight under optimal conditions prior to counting. To review, the 30 min sample were allowed to grow for 30 min. Then PVP-I was added to the appropriate sample and material was removed for plating after 5 min. The 6 hr samples grew for 6 hr prior to the PVP-I addition followed by plating; the 24 hr samples grew for 24 hr prior to PVP-I addition.

Medium	30 min	6 hr	24 hr
Broth + 2% citrate	3+	4+	4+
Broth + PVP-I	3+	3+	4+
Broth + 2% citrate + PVP-I	1+	NG	NG

These results suggest that citrate potentiates the killing power of PVP-I and/or cells grown in the presence of citrate were weakened and made more susceptible to the iodine.

Citrate Effect on Iodine Based Viral Inactivation

The following experiments represent a refinement of initial iodine experiments that resulted in the discovery of the citrate effect. The iodine inactivation process is the result of many years of investigations. The general idea is to expose a sample of virally contaminated solution (here human plasma) to a source of active iodine and then remove the iodine before damage is done to delicate proteins. In the case of plasma it is possible to monitor sensitive blood clotting enzymes to check for protein denaturation. The process that has been developed involves using an iodine containing resin to donate iodine to the solution and an iodine/iodide binding resin to remove the iodine and any resulting iodide. A surprising development of this line of research was that repeated passage through a mixed column of iodine donating and iodine/iodide absorbing resin was especially effective at inactivating virus while retaining activity of blood clotting enzymes. That work is now U.S. Patent No. 6,045,787 which is incorporated herein by reference.

In the present experiment a 1500 ml sample of human plasma was spike with approximately 1×10^6 particles of Porcine Parvo Virus (PPV) and 5×10^5 particles of Bovine Viral Diarrhea Virus (BVDV). The plasma was then brought to 8% sodium citrate.

The plasma was then cycled four times through a large column of iodine and iodine capture mixed resin (1:8). After each pass residual iodine (I_2) and iodide (I^-) were measured using an iodide sensitive electrode (Orion Research Instruments). The electrode reads the iodide concentration directly. Sodium ascorbate was added to each sample to determine iodine concentration. Any increase in iodide reading represented reduction of iodine to iodide by the ascorbate. It was not expected that much if any iodine or iodide would be present because of the large excess of iodine/iodide binding resin in the column.

Iodide and Iodine per Pass

Treatment	Iodide (ppm)	Iodine (ppm)
Control (no column treatment)	0	0
Pass 1	0	0
Pass 2	1	0
Pass 3	0	0
Pass 4	0	0

The concentration of the various viruses was determined using a Viral Endpoint Assay (VEPA). Briefly, samples to be tested were placed on a monolayer of appropriate cells (*i.e.*, host cells for the particular virus). After an incubation period, virus presence was determined by plaque formation. Each sample was tested over a range of serial two-fold dilutions so that the greatest dilution still showing plaque formation could be determined. Viral titer was calculated from this dilution assay using a well known formula.

As had been determined from past research, the column treatment resulted in some type of latent damage to the virus. It was found that holding the sample for up to 24 hrs prior to plating onto a cell culture would result in increased viral inactivation.

PPV Inactivation

Time		1	2	3	4	5	6	7	8	9	10	11	12	<u>Titer</u>
	control	4	4	4	4	4	4	4	4	2	0	0	0	6.3
0 hrs	P1	4	4	4	4	4	4	4	1	0	0	0	0	5.4

	P2	4	4	4	4	4	0	0	0	0	0	0	0	3.8
	P3	4	4	4	2	0	0	0	0	0	0	0	0	2.8
	P4	4	2	0	0	0	0	0	0	0	0	0	0	1.4
6 hrs	P1	4	4	4	4	3	0	0	0	0	0	0	0	3.7
	P2	4	4	4	2	0	0	0	0	0	0	0	0	2.8
	P3	4	3	0	0	0	0	0	0	0	0	0	0	1.6
	P4	2	0	0	0	0	0	0	0	0	0	0	0	0.7
24 hrs	P1	4	1	0	0	0	0	0	0	0	0	0	0	1.2
	P2	0	0	0	0	0	0	0	0	0	0	0	0	0
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0

BVDV Inactivation

Time		1	2	3	4	5	6	7	8	9	10	11	12	<u>Titer</u>
	control	4	4	4	4	4	4	4	1	0	0	0	0	5.4
0 hrs	P1	4	4	4	4	0	0	0	0	0	0	0	0	3.1
	P2	4	4	3	0	0	0	0	0	0	0	0	0	2.3
	P3	4	4	0	0	0	0	0	0	0	0	0	0	1.7
	P4	2	0	0	0	0	0	0	0	0	0	0	0	0.7

6 hrs	P1	4	4	0	0	0	0	0	0	0	0	0	0	0	1.7
	P2	4	2	0	0	0	0	0	0	0	0	0	0	0	1.4
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24 hrs	P1	3	0	0	0	0	0	0	0	0	0	0	0	0	.9
	P2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0	0

These results show that BVDV is somewhat more sensitive than PPV, and that both viruses show increased kill if the samples are incubated for up to 24 hrs before being plated on the cell culture. In earlier experiments without citrate it was necessary to do at least six passes and incubate for 24 hrs to get a complete kill of these very resistant viruses. Also, although the resin used for iodine/iodide capture also has some affinity for citrate, citrate does not appear to interfere with the capture of iodine/iodide. It seems likely that all of the citrate sites become saturated during the first pass. Thereafter iodide merely exchanges for citrate during the capture process.

The above experiment was repeated with a 2000 ml plasma sample with the addition of a 2% sodium citrate sample to the 8% sodium citrate sample. Also, samples were incubated up to 48 hrs prior to plating on the VEPA cell assay.

Results with the iodide/iodine measurement were essentially as before. T

Iodide and Iodine per Pass

Treatment	Iodide (ppm)	Iodine (ppm)
Control (2% Citrate) (no column treatment)	0	0
Pass 1 (2% Citrate)	0	0
Pass 2 (2% Citrate)	0	0
Pass 3 (2% Citrate)	0	0
Pass 4 (2% Citrate)	1	0
Pass 1 (4% Citrate)	0	0
Pass 2 (4% Citrate)	0	1
Pass 3 (4% Citrate)	0	0
Pass 4 (4% Citrate)	0	0

PPV Inactivation (8% Citrate)

Time		1	2	3	4	5	6	7	8	9	10	11	12	<u>Titer</u>
	control	4	4	4	4	4	4	4	4	3	0	0	0	6.5
0 hrs	P1	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P2	4	4	4	4	4	3	0	0	0	0	0	0	4.4
	P3	4	4	4	2	0	0	0	0	0	0	0	0	2.8
	P4	4	4	0	0	0	0	0	0	0	0	0	0	1.7
6 hrs	P1	4	4	4	4	4	0	0	0	0	0	0	0	3.8
	P2	4	4	3	1	0	0	0	0	0	0	0	0	2.4

	P3	4	2	0	0	0	0	0	0	0	0	0	0	0	1.4
	P4	3	0	0	0	0	0	0	0	0	0	0	0	0	0.9
24 hrs	P1	4	2	0	0	0	0	0	0	0	0	0	0	0	1.4
	P2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48 hrs	P1	4	0	0	0	0	0	0	0	0	0	0	0	0	.7
		0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0	0	0

BVDV Inactivation (8% Citrate)

Time		1	2	3	4	5	6	7	8	9	10	11	12	<u>Titer</u>
	control	4	4	4	4	4	4	4	0	0	0	0	0	5.2
0 hrs	P1	4	4	4	4	2	0	0	0	0	0	0	0	3.5
	P2	4	4	0	0	0	0	0	0	0	0	0	0	1.7
	P3	4	2	0	0	0	0	0	0	0	0	0	0	1.4
	P4	1	0	0	0	0	0	0	0	0	0	0	0	0.4
6 hrs	P1	4	4	1	0	0	0	0	0	0	0	0	0	1.8

	P2	4	1	0	0	0	0	0	0	0	0	0	0	1.1
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0
24 hrs	P1	4	1	0	0	0	0	0	0	0	0	0	0	1.1
	P2	0	0	0	0	0	0	0	0	0	0	0	0	0
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0
48 hrs	P1	0	0	0	0	0	0	0	0	0	0	0	0	0
	P2	0	0	0	0	0	0	0	0	0	0	0	0	0
	P3	0	0	0	0	0	0	0	0	0	0	0	0	0
	P4	0	0	0	0	0	0	0	0	0	0	0	0	0

PPV Inactivation (2% Citrate)

Time		1	2	3	4	5	6	7	8	9	10	11	12	<u>Titer</u>
	control	4	4	4	4	4	4	4	4	1	0	0	0	6.0
0 hrs	P1	4	4	4	4	4	4	4	4	0	0	0	0	5.9
	P2	4	4	4	4	4	4	4	4	0	0	0	0	5.9
	P3	4	4	4	4	4	4	4	4	2	0	0	0	6.3
	P4	4	4	4	4	4	4	4	4	0	0	0	0	5.9

6 hrs	P1	4	4	4	4	4	4	4	4	1	0	0	0	6.0
	P2	4	4	4	4	4	4	4	4	0	0	0	0	5.9
	P3	4	4	4	4	4	4	4	2	0	0	0	0	5.6
	P4	4	4	4	4	4	4	4	0	0	0	0	0	5.2
24 hrs	P1	4	4	4	4	4	4	4	3	0	0	0	0	5.8
	P2	4	4	4	4	4	4	1	0	0	0	0	0	4.7
	P3	4	4	4	4	4	0	0	0	0	0	0	0	3.8
	P4	4	4	4	4	2	0	0	0	0	0	0	0	3.5
48 hrs	P1	4	4	4	4	4	4	4	4	0	0	0	0	5.9
		4	4	4	4	4	4	0	0	0	0	0	0	4.5
		4	4	4	4	2	0	0	0	0	0	0	0	3.5
		4	2	0	0	0	0	0	0	0	0	0	0	1.4

BVDV Inactivation (2% Citrate)

Time		1	2	3	4	5	6	7	8	9	10	11	12	<u>Titer</u>
	control	4	4	4	4	4	4	3	0	0	0	0	0	5.1
0 hrs	P1	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P2	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P3	4	4	4	4	4	4	3	0	0	0	0	0	5.1

	P4	4	4	4	4	4	4	4	0	0	0	0	0	5.2
6 hrs	P1	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P2	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P3	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P4	4	4	4	4	4	4	3	0	0	0	0	0	5.1
24 hrs	P1	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P2	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P3	4	4	4	4	4	4	0	0	0	0	0	0	4.5
	P4	4	4	4	4	4	2	0	0	0	0	0	0	4.2
48 hrs	P1	4	4	4	4	4	4	4	0	0	0	0	0	5.2
	P2	4	4	4	4	4	4	0	0	0	0	0	0	4.5
	P3	4	4	4	4	4	0	0	0	0	0	0	0	3.8
	P4	4	4	4	4	2	0	0	0	0	0	0	0	3.5

These results show that 8% citrate is dramatically more effective than 2% citrate. The citrate results are relatively paradoxical in that a mixture of citrate and povidone iodine will result in iodide formation presumably from the oxidation of the citrate by the iodine. That would lead one to expect that added citrate would result in blocking of the iodine disinfection as the citrate became oxidized instead of the viral proteins or nucleic acids. One possibility is that the oxidized citrate provides some type of activated intermediate compound that preferentially reacts with the viral components. If this is the case, this

intermediate must react solely with nucleic acids since clotting enzyme assays (not shown) demonstrate that the increased citrate concentration does not result in increased protein denaturation. Preliminary results indicate that there may be benefit to preincubating with the citrate prior to addition of iodine or other
5 disinfecting agent.

It is tempting to imagine that there is a unified pathway that explains all of the citrate effects. However it is very difficult to see what this might be. Citric acid is an important part of the Tricarboxylic acid pathway of oxidative metabolism. It is known that citrate is generally rapidly metabolized. Perhaps
10 with bacteria the excess citrate results in some type of metabolic imbalance that make the bacteria especially susceptible to antibiotics and disinfecting dyes. Also, most bacteria have active membrane transport systems for taking up metabolites such as citrate. Perhaps the same transport process that takes up citrate results in an accumulation of antibiotics, dyes or plant products (like the wine
15 polyphenols) in the cell. This might explain the citrate effect on antibiotic resistant microorganisms.

Whatever the mechanism the carboxylic acid effect should be a boon to treatment of drug resistant infections and extending the life of blood fractions such as platelets. Citrate enhancement of antibiotics may work intravenously
20 although the chelating effects of citrate as well as the rapid metabolism of citrate may preclude this use. However, citrate is ideal for use in dialysis procedures to prevent accidental infection. Citrate enhancement is also ideal for use in ointments and other topical applications of anti-infective agents. Citrate enhancement of antibiotics should prove especially effective as antibacterial
25 washes for burn patients. With biologics such as blood and blood products the excess citrate can readily be removed by ion exchange or supplemental calcium can be added to overcome the chelation. In combination with mixed iodine/capture resin columns citrate provides a dramatic improvement in the

destruction of contaminating viruses while having a negligible effect on labile proteins. It seems likely that the enhanced killing powers attributed to citrate addition will permit the inactivation of prions and other still mysterious pathogens.

5 The present invention is described in terms of "citrate" or dicarboxylic acids. The exact mechanism of the present invention is not yet understood. The effect does not seem to extend to all dicarboxylic acids as tartaric acid appears to be relatively ineffective. Other analogous carboxylic acids such as isocitric acid are being investigated.

10 The following claims are thus to be understood to include what is specifically illustrated and described above, what is conceptually equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention. Those skilled in the art will appreciate that various adaptations and modifications of the just-described preferred
15 embodiment can be configured without departing from the scope of the invention. The illustrated embodiment has been set forth only for the purposes of example and that should not be taken as limiting the invention. Therefore, it is to be understood that, within the scope of the appended claims, the invention may be practiced other than as specifically described herein.

20

I claim:

1 1. A method for enhancing the effectiveness of antimicrobial agents
2 comprising the step of combining the antimicrobial agent with citric acid and or salts or citric
3 acid.

1 2. The method of Claim 1, wherein the antimicrobial agent is selected
2 from the group consisting of antibiotics, povidone iodine, iodine, polyphenols of plant origin,
3 and disinfectant organic dyes.

1 3. The method of Claim 2, wherein the disinfectant organic dyes are
2 selected from the group consisting of methylene blue and crystal violet.

1 4. A method for extending the life of a platelet concentrate and preventing
2 the multiplication of bacteria therein comprising the step of adding between 1 and 15% by
3 weight of sodium citrate to the platelet concentrate.

4 5. The method of Claim 4, further comprising the step of removing the
5 sodium citrate.

1 6. The method of Claim 4, further comprising the step of adding an
2 antimicrobial agent to the platelet concentrate.

1 7. The method of Claim 4, wherein the antimicrobial agent is selected
2 from the group consisting of antibiotics, povidone iodine, iodine, polyphenols of plant origin,
3 and disinfectant organic dyes.

1 8. The method of Claim 7, wherein the disinfectant organic dyes are
2 selected from the group consisting of methylene blue and crystal violet.

1 9. A method for disinfecting solutions containing red blood cells
2 comprising the step of adding between 1 and 15% by weight of sodium citrate to the solution.

1 10. The method of Claim 9, further comprising the step of removing the
2 sodium citrate.

1 11. The method of Claim 9, further comprising the step of adding an
2 antimicrobial agent to the solution.

1 12. The method of Claim 9, wherein the antimicrobial agent is selected
2 from the group consisting of antibiotics, povidone iodine, iodine, polyphenols of plant origin,
3 and disinfectant organic dyes.

1 13. The method of Claim 12, wherein the disinfectant organic dyes are
2 selected from the group consisting of methylene blue and crystal violet.

1 14. A topical preparation comprising an antimicrobial agent and a
2 concentration of citrate sufficient to enhance an antimicrobial action of the antimicrobial
3 agent.

1 15. The preparation of Claim 14, wherein the antimicrobial agent is
2 selected from the group consisting of antibiotics, povidone iodine, iodine, polyphenols of
3 plant origin, and disinfectant organic dyes.

1 16. The preparation of Claim 15, wherein the disinfectant organic dyes are
2 selected from the group consisting of methylene blue and crystal violet.

ABSTRACT

Simple carboxylic acids, in particular dicarboxylic acids such as citric acid shows an unexpected ability to enhance the antimicrobial power of a wide range of disinfectant and/or antibiotic agents. As little as 1% citrate greatly enhances the ability of antibiotics to kill or inhibit a wide range of bacterial species including antibiotic resistant strains. Citrate alone is effective in preventing bacterial growth in platelet concentrates and in red blood cell suspensions. Effective concentrations of citrate cause little if any damage to blood cells.

Besides enhancing the power of antibiotics citrate also enhances the antimicrobial properties of disinfectant organic dyes such as crystal violet and methylene blue. In addition citrate enhances the antimicrobial properties of polyphenols of plant origin. Iodine-based disinfectants are also enhanced without enhancing protein denaturation.